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Use of Real-Time PCR To Process the First Galactomannan-Positive Serum Sample in Diagnosing Invasive Aspergillosis

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Positive galactomannan (GM) antigenemias are included as a microbiological item in the diagnosis of probable or possible invasive aspergillosis (IA). Because false-positive GM results frequently occur, at least two positive results on two different samples are required. Waiting for clinical specimens can delay the initiation of treatment. As an alternative, we wondered whether detection of circulating *Aspergillus* DNA on the first positive GM serum sample could aid in diagnosing IA. Therefore, we retrospectively screened the first GM-positive serum samples from 29 patients from our hematology unit for *Aspergillus* DNA using real-time PCR. We compared the real-time PCR results with the final classification of proven, probable, and possible IA according to consensual criteria. No clear correlation between PCR results and the classification with the medical files could be shown. However, a positive PCR result was associated with a poor prognosis (Fisher's test; $P = 0.01$). Our preliminary data suggest that a positive PCR result could indicate a more advanced stage of the disease. Therefore, concomitant positive PCR and GM results may justify the initiation of antifungal therapy in neutropenic patients. In contrast, a negative PCR on the first positive GM sample may argue for postponing costly antifungal administration until additional arguments for the diagnosis of IA are presented.

Invasive aspergillosis (IA) is the main cause of mortality due to infection in leukemic patients and allogeneic stem cell transplant recipients (21). The average death rate is approximately 50% in leukemic patients and 90% in allogeneic bone marrow transplant recipients (14). This poor prognosis is due in part to a failure to diagnose early, which in turn results in delays in initiating antifungal therapy.

Definitions of proven, probable, and possible IA, based on a combination of host factors and radiological, clinical, and biological data, have been recently proposed so that homogeneous groups of patients can be formed for clinical research (3). In these consensual proposals, positive galactomannan (GM) antigenemia has been included as a microbiological criterion for probable or possible diagnosis. This has been mainly based on the reported 90% sensitivity of the Platelia *Aspergillus* test (Bio-Rad, Marnes-la-Coquette, France) (17). It is now a common practice in Europe, especially in hematological units, to screen patients at risk for IA twice a week (23). A positive GM result in a neutropenic patient frequently triggers antifungal therapy, as a positive GM result has been reported to appear before clinical signs (18, 25, 27).

However, false-positive GM results are frequent; GM has thus been used as a microbiological criterion only when two separate serum samples are positive (3). This procedure has led to an improvement in the predictive positive value, although the rate of false-positive results remains high, between 10 and 15% (7, 19). This problem of false positives has been recently exacerbated because of GM contamination of samples from patients treated with some piperacillin-tazobactam and amoxicillin-clavulanic acid batches (1, 22, 28–30). False-positive GM results could also be due to the digestive absorption of galactomannan in food, as well as some bacterial membrane-associated molecules (2, 23, 24).

This underlines the necessity for testing more than one sample. However, the wait for additional samples can delay the initiation of treatment. Instead of waiting for more samples, another test could be performed on the same sample to improve the specificity of the GM test.

Aspergillus DNA is another possible biological marker for the diagnosis of IA (6, 10–12). However, the international consensus defining invasive fungal infections (IFIs) in oncohematological patients does not include PCR as a diagnostic tool, because of divergent results due to the lack of standardization (3). Some of the shortcomings of the standard PCR assays are eliminated by real-time PCR assays (5). This new technology has been adapted for the detection of *Aspergillus* DNA (8, 9, 16, 26).

We wondered whether detecting circulating *Aspergillus* DNA in the first positive GM sample by real-time PCR could help in diagnosing IA in hematological patients. In an emergency diagnosis of IA, a real-time PCR performed on the same serum sample could shorten the time response and help in decision making. Therefore, we retrospectively screened the first GM-positive serum sample, collected as part of our routine practice and stored at −20°C, for *Aspergillus* DNA by real-time PCR.

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MATERIALS AND METHODS

Thirty-one consecutive patients from the hematology unit of Besançon University Hospital were studied between January 2001 and December 2002. The inclusion criteria were the presence of risk factors for IA, as they are defined by the National Institute of Allergy and Infectious Diseases Mycoses Study Group and the European Organisation for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group (MSG/EORTC) consensus (neutropenia is defined as an absolute neutrophil count below 0.5×10^9/liter, and/or persistent fever under broad-spectrum antibiotherapy, and/or recent or current use of immunosuppressive agents or corticosteroids) and one positive GM result.

The GM test was performed using the Platelia *Aspergillus* sandwich enzyme-linked immunosorbent assay (Bio-Rad). A GM test was positive when the index was ≥1.5 (following the manufacturer's recommendations). Serum samples were tested as part of the twice-weekly systematic surveillance of all the hospitalized patients with host factors; it was tested once weekly for the other patients (outpatients). After testing, 1.5 ml of serum was aliquoted and stored at −20°C. Clinical signs were recorded in the following 90 days. Final analysis of the clinical files and charts was done at the death of the patient or at day 90, and classification as possible, probable, and proven IA was performed by using MSG/EORTC consensual definitions (3).

For each patient, only the first positive GM serum sample was retrospectively analyzed by real-time PCR with a Light Cycler instrument (Roche Molecular Biochemicals, Meylan, France) as previously described (9). Briefly, DNA extraction was performed using the High Pure PCR template kit (Roche Molecular Biochemicals). We used a starting volume of 200 μl of serum, eluted in 50 μl of elution buffer. PCR was performed with 5 μl of DNA extract. Mitochondrial DNA from *Aspergillus fumigatus* and *Aspergillus flavus* was detected by using previously described primers and probes (9). Fluorescence curves were analyzed with Light Cycler software, version 3.5. Quantitative results were expressed by determination of the threshold of detection, or crossing point (Cp), which marked the cycle at which fluorescence of the sample became significantly different from the baseline signal. Thus, the higher the Cp value was, the smaller the amount of DNA in the sample was. Two independent DNA extractions were performed for each serum. Each DNA extract was submitted to PCR amplification. Thus, two Cp results, one for each replicate, were obtained for each serum sample tested. Two analyses of the results were performed: one analysis designated a sample positive only when the Cp value was ≤40 cycles in both replicates; the second analysis designated a sample positive when at least one replicate had a Cp value of ≤43 cycles. One positive control, using *A. fumigatus* DNA, and two negative controls were added to each run. The negative controls were two sterile water samples, one submitted to the extraction and amplification protocols and the other submitted only to the amplification protocol. This was done to verify that there was no contamination in any step of the technical procedure. Strict measures were taken to prevent DNA contamination. DNA extractions were performed inside a biological safety cabinet. Contamination with previously amplified products was prevented by the systematic use of uracil-*N*-glycosylase.

RESULTS

Classification of the 31 patients according to the consensual MSG/EORTC criteria was as follows: 1 patient had proven scedosporiosis, 1 patient had proven candidiasis, 1 patient had proven IA, 6 patients had probable IAs, 11 patients had possible IAs, and 11 patients had no IFI. Samples from the patient with candidiasis and the patient with scedosporosis were PCR negative and were excluded from further analysis because these infections were due to species that were not targeted by the primers or the probe used. Table 1 shows clinical and microbiological data from the 29 other patients.

These 29 patients had a first positive GM sample (one inclusion criterion). For four of these patients, no additional sera were tested for GM because of patient death or transfer to another unit. A second serum sample was available for GM testing for the 25 other patients. Fourteen of the patients had a second positive sample, which led to the inclusion of this result as a microbiological criterion for the final classification of IA. Thus, for 11 (44%) of the 25 patients who had a second test, the first GM result was considered a false positive; these 11 patients were non-IFI in the final classification. No PCR test was performed on these additional samples, as our study was not designed to compare the parallel kinetics of GM and DNA. Amplifications were performed in 15 different runs. Each run included a negative extraction control, a negative amplification control, a positive control, and 10 serum samples. No PCR-positive result was observed in the negative controls. A dilution of purified DNA of *A. fumigatus* (2.5 fg/μl) was used as a positive control and consistently gave a Cp value of 36.3 (± 0.5) cycles. The Cp values for the clinical samples were always >36 cycles, i.e., equivalent to a DNA concentration of <2.5 fg/μl of serum.

When a result was considered positive only when the Cp value was ≤40 cycles in both replicates, samples from only four patients (patient numbers 1, 2, 4, and 6) (Table 1) were PCR positive. All four of the patients were classified as having proven, probable, or possible IA (Fig. 1A). With this analysis of PCR results, the association between probable-proven cases and positive PCR results was significant (Fisher's test; $P = 0.033$).

When a result was considered positive when at least one replicate had a Cp value of ≤43 cycles, 13 patient samples were PCR positive and 16 were PCR negative. No significant association between PCR results and final classification of IA was observed, especially because 4 of the 11 non-IA patients had samples that were PCR positive (Fig. 1B). However, a positive PCR was significantly associated with a fatal outcome, as all the 16 patients with PCR-negative samples had a favorable outcome, while 5 out of 13 PCR-positive patients died (Fisher's test; $P = 0.01$). Radiological and/or clinical signs were recorded for these five patients in the final classification.

Given the reported high rate of false-positive GM results, some, but not all of the GM-positive patients were given anti-*Aspergillus* antifungals intravenously (i.v.) at the time of the first positive GM result. Seven of the 13 PCR-positive patients were given antifungals. Fourteen of the 16 PCR-negative patients were not given antifungals. These decisions were made with no knowledge of the PCR results, due to the retrospective design of the study. When PCR results were compared to the final analysis, if the 13 PCR-positive patients had been given antifungals, four additional treatments would have been prescribed to non-IFI patients. These patients were not neutropenic and had no clinical signs at the time of the first positive GM. However, two additional patients with possible IA would have been given antifungals. Both died having received no antifungal therapy. By contrast, negative PCR results would have lent further support to the decision not to administer antifungals, a decision that was made at the time of the first serum sampling for the 14 patients with positive GM. At final analysis, these 14 patients were classified as non-IFI ($n = 7$), possible IA ($n = 6$), and probable IA ($n = 1$). For all of them, the outcome was favorable with no anti-*Aspergillus* therapy.

DISCUSSION

In this pilot study, we were unable to show a clear correlation between a real-time PCR result at the time of the first GM-positive test and the final classification of IA using the consensual definitions (3). The lack of correlation could be linked to the high rate of false-GM-positive results. In our

TABLE 1. Final analysis of the clinical files at day 90 of follow-up from 29 patients with host factors and a first positive GM sample

Patient no.	Age (yr)	Underlying disease[a]	Clinical criterion[c]		Microbiological criterion[c]		i.v. antibiotic(s)[g]	Anti-*Aspergillus* i.v. antifungal(s)[c]	Outcome at day 90 or day (D) of death	Classification at final analysis
			Respiratory signs	Radiologic signs	≥2 GM-positive sera	PCR				
1	67	MM	+	+	Yes	Pos◆	TZP	+	D75	Proven IA[d]
2	60	MM	+	+	Yes	Pos◆	AMK-TEC-FEP	+	D20	Probable IA
3	67	AML[b]	+	+	Yes	Pos		+	Alive	Probable IA
4	2	AML[b]	+	+	Yes	Pos◆	IPM-CIP	+	Alive	Probable IA
5	11	SAA	+	+	No	Pos	TZP	+	D5	Possible IA
6	49	AML	+	+	No	Pos◆	IPM-AMK-VAN-ERY	−	D3	Possible IA
7	49	NHL	+	−	No	Pos	FEP-VAN	−	D1	Possible IA
8	51	NHL	−	−	Yes	Pos	TZP	+	Alive	Possible IA
9	44	AML	−	−	Yes	Pos	TZP-VAN	+	Alive	Possible IA
10	35	ALL	−	−	No	Pos		−	Alive	No IFI
11	38	ALL	−	−	No	Pos		−	Alive	No IFI
12	17	ALL	−	−	No	Pos	CAZ-VAN-CIP	−	Alive	No IFI
13	18	ALL	−	−	No	Pos		−	Alive	No IFI
14	63	CML	+	+	Yes	Neg	CAZ-TEC	+	Alive	Probable IA[e]
15	51	AML	+	+	Yes	Neg	AMC-VAN-CIP	+	Alive	Probable IA
16	51	ALL	+	+	No	Neg	CRO-TOB	−	Alive	Probable IA[f]
17	59	MM	−	−	Yes	Neg	FEP-AMK	−	Alive	Possible IA
18	45	CLL	−	−	Yes	Neg		−	Alive	Possible IA
19	60	NHL	−	−	Yes	Neg	TZP-TOB	−	Alive	Possible IA
20	51	NHL	−	−	Yes	Neg	TZP-TOB	−	Alive	Possible IA
21	62	MM	−	−	Yes	Neg		−	Alive	Possible IA
22	59	NHL	−	−	Yes	Neg	AMX-SXT	−	Alive	Possible IA
23	12	ALL	−	−	No	Neg		−	Alive	No IFI
24	45	ALL	−	−	No	Neg		−	Alive	No IFI
25	41	NHL	−	−	No	Neg		−	Alive	No IFI
26	22	ALL	−	−	No	Neg	TZP	−	Alive	No IFI
27	58	AML	−	−	No	Neg		−	Alive	No IFI
28	48	RCC	−	−	No	Neg		−	Alive	No IFI
29	59	MM	−	−	No	Neg	TZP-OFX-VAN	−	Alive	No IFI

[a] MM, multiple myeloma; AML, acute myeloblastic leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia; NHL, non-Hodgkin's lymphoma; CLL, chronic lymphocytic leukemia; MAS, macrophage activation syndrome; RCC, renal cell carcinoma; SAA, severe aplastic anemia.

[b] Patient had an allograft.

[c] +, present; −, absent; Neg, negative; Pos, positive when at least one replicate had a Cp value of ≤43; Pos◆, positive with both replicates with a Cp value of ≤40 cycles.

[d] *A. fumigatus* in lung biopsy.

[e] *A. fumigatus* in bronchoalveolar lavage.

[f] GM in bronchoalveolor lavage.

[g] AMK, amikacin; AMX, amoxicillin; AMC, amoxicillin-clavulanic acid; FEP, cefepim; CAZ, ceftazidim; CRO, ceftriaxone; CIP, ciprofloxacin; ERY, erythromycin; IPM, imipenem; OFX, ofloxacin; TZP, piperacillin-tazobactam; TEC, teicoplanin; TOB, tobramycin; SXT, trimethoprim-sulfamethoxazole; VAN, vancomycin.

study, the false-positive rate was 44% when only the first test was taken into account; this is much higher than what the literature reports. The false-positive rate could have been even higher if a lower threshold had been retained, as suggested by some authors (20). Moreover, eight patients without patent clinical signs of IA were classified as possible IA due to two GM-positive results, and they all had a favorable outcome with no anti-*Aspergillus* therapy. This underlines the recently observed deleterious effects of GM contamination of antibiotic batches (1, 22, 28–30). Indeed, 3 of the 11 non-IFI patients and 9 of the 11 patients classified as possible IA patients were receiving antibiotics at the time of the first GM serum sampling (8 of these 12 patients were receiving piperacillin-tazobactam or amoxicillin-clavulanic acid).

Another explanation for the lack of correlation between PCR results and the final classification could be the false positives from the PCR assay. The main advantage of real-time PCR technology is the low risk of false-positive results, due to contamination from the environment (5). However, real-time PCR does not eliminate the risk of contamination of reagents by fungal DNA (15). In the present study, we used commercial DNA extraction kits, and all of our negative controls were negative. Therefore, the contamination of the reagents used with *Aspergillus* DNA is unlikely. In contrast, fungal DNA contamination from antibiotics could occur in the same way that GM contamination could (1, 22, 28, 29). To deal with this problem, the time of the blood draw should be recorded and compared to the administration of i.v. antibiotics, and GM and PCR testing of every batch of antibiotics should be performed (which would be difficult to implement on a routine basis).

An intriguing point in our study is the fact that the amount of DNA, as estimated from the Cp values, was very small in all the PCR-positive patients. This had already been reported when quantitative PCR assays were used (8, 9, 16). Consequently, several problems in interpreting real-time PCR were encountered. First, quantification of very small DNA amounts limits the reproducibility of real-time PCR results. The DNA concentrations calculated after the positive control never exceeded a few femtograms per microliter. According to Poisson's law, PCR results cannot be consistently positive at these

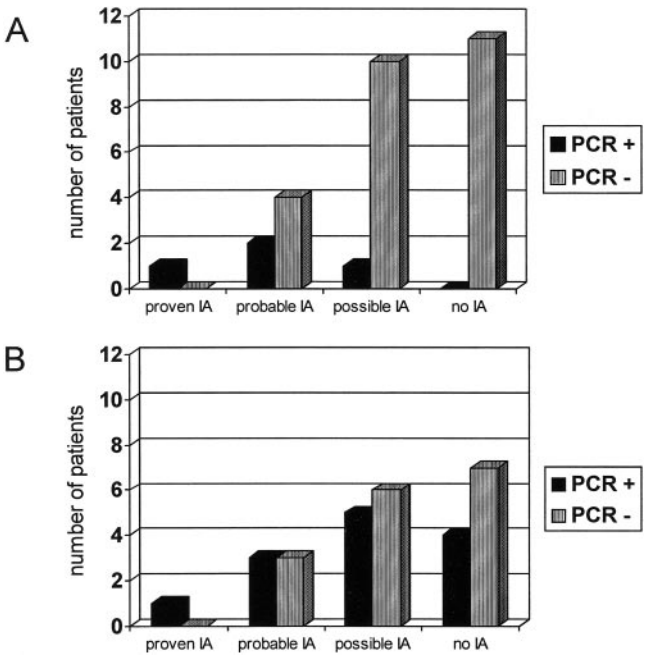

FIG. 1. Correlation between PCR results and final classification, according to consensual definition, for 29 patients at risk for IA. (A) Results were considered positive only when Cp values were ≤40 cycles in both replicates (significant association of positive results with proven and probable cases [Fisher's test; $P = 0.033$]). (B) Results were considered positive when at least one replicate had a Cp value of ≤43 cycles (no significant association).

very low concentrations. Thus, one replicate can be positive and the other one can be negative, depending on whether the target DNA is present or not in the volume tested. For decision making, we suggest considering a sample as positive even if only one replicate is <43 cycles. In our retrospective study, two patients had only one positive PCR replicate and both died within 5 days; one of them did not receive i.v. antifungal therapy.

The small DNA amounts prevent any analysis of correlation between DNA and GM quantification. A larger DNA burden could indicate more advanced disease, as recently suggested, with the use of a different *Aspergillus* DNA quantification assay (31). In our study, some patients had very small DNA burdens, even in the presence of high GM levels and clinical and/or radiological signs and even just before death. The fact that PCR is less sensitive than GM in humans (6, 13) and animals (4) has already been reported. On the other hand, when the Cp value was ≤40 cycles in both replicates, the patients were all classified as IA. This suggests that the larger the DNA amount, the more probable the diagnosis of IA.

Indeed, one of the main points of our study is that a PCR-positive result, when obtained on the first GM-positive serum sample from patients at risk for IA, was associated with a poor prognosis. Clinical and radiological signs, also associated with a poor prognosis, have been reported to appear after (median, 6 days) the first GM result in more than half of the cases (18). Thus, in decision making, a PCR-positive result could reinforce the decision to initiate antifungal therapy, with the understanding that the therapy could be stopped if diagnosis of IA was not confirmed. In contrast, a negative PCR result could argue in favor of postponing costly treatment with potential side effects until additional arguments favoring therapy were presented. However, a PCR-negative result must be interpreted with caution, because our real-time PCR targets *A. fumigatus* and *A. flavus* and other molds could be involved. These preliminary data need to be confirmed by a prospective study with a larger cohort.

Our study suggests that performing real-time PCR as soon as the first serum sample is positive for GM can be of interest, not to improve diagnosis of IA but as a help in decision making with patients hospitalized in a hematology department and at risk for invasive fungal infections. Our results also underline the difficulties in interpreting the meaning of biological tests in the absence of definite standards. For prospective studies, our data suggest the need to record antibiotic therapy and possibly the time of the venous punctures and compare them to the results of GM and PCR testing.

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